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**W-5000 Köln 1(DE)**(54) **DNA encoding acylamino acid racemase and its use.**

(57) The present invention relates to a DNA fragment containing a gene encoding acylamino acid racemase. The acylamino acid racemase can be produced industrially at high efficiency using the DNA fragment.

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## FIELD OF THE INVENTION

This invention relates to a fermentative method of producing acylamino acid racemase, to a novel microorganism useful in said method and to a novel DNA fragment useful in producing said novel microorganism.

## BACKGROUND OF THE INVENTION

Acylamino acid racemase is widely distributed among actinomycetes. It is a specific enzyme which catalyzes racemization of optically active N-acylamino acids but does not act on optically active amino acids and its properties have already been made clear in detail (US Patent No. 4,981,799; Abstracts of Papers presented at the 1990 Annual Meeting of the Japan Society for Bioscience, Biotechnology, and Agrochemistry, page 368, 1990).

As mentioned above, acylamino acid racemase is a useful enzyme utilizable in the production of optically active amino acids. However, any satisfactory process for producing said enzyme at low cost and in an efficient manner has not been developed as yet and, accordingly, the advent of a more advantageous method has been awaited.

Under these circumstances, the present inventors made intensive investigations and made a report on acylamino acid racemase produced by *Amycolatopsis* sp. TS-1-60 (Abstracts of Papers presented at the 1990 Annual Meeting of the Japan Society for Bioscience, Biotechnology, and Agrochemistry, page 368, 1990). Furthermore they succeeded in cloning a DNA encoding said acylamino acid racemase from *Amycolatopsis* sp. TS-1-60 and, after further investigations, they have now completed the present invention.

## SUMMARY OF THE INVENTION

The present invention provides a DNA fragment containing a gene encoding acylamino acid racemase, a vector with said DNA fragment inserted therein, a novel microorganism transformed with said vector and capable of producing acylamino acid racemase, and a method of producing acylamino acid racemase which comprises using said microorganism.

## BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows the base sequence of a gene encoding acylamino acid racemase as determined in Example 3; Fig. 2 shows a restriction enzyme cleavage map of the DNA fragment containing the gene encoding acylamino acid racemase obtained in Example 1; Fig. 3 shows a process for preparing the plasmid pRA4; Fig. 4 shows a process for preparing the plasmid pRA7; Fig. 5 shows a process for preparing the plasmid pRA4A; Fig. 6 shows a process for producing the plasmid pRA7A; Fig. 7 shows a process for producing plasmid pRA4N; Fig. 8 shows a process for producing plasmid pRA4NB; and Fig. 9 shows a process for producing plasmid pET-3cN.

## DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

The DNA fragment according to the invention which contains a gene encoding acylamino acid racemase (hereinafter sometimes referred to also as acylamino acid racemase-encoding DNA or simply as acylamino acid racemase DNA) has been discovered for the first time by the present inventors and can be produced, by isolating the relevant DNA from the acylamino acid racemase producing actinomycetes, for example, *Streptomyces* sp. Y-53 (IFO 14596; FERM P-9518) or *Amycolatopsis* sp. TS-1-60 (IFO 15079; FERM BP-3092) and others, inserting said DNA into a phage or plasmid, transforming a host with the resulting recombinant phage or plasmid, cultivating the transformants obtained, isolating a phage or plasmid containing the desired DNA from among the transformants by an appropriate method, for example by immunoassay using an acylamino acid racemase antibody or by plaque or colony hybridization using a DNA probe, excising the desired cloned DNA from the phage or plasmid and subcloning said cloned DNA into an appropriate plasmid. The thus-obtained DNA can be inserted into a plasmid or phage and the resulting recombinant can be used for the transformation of a host, for example *Escherichia coli*, an actinomycete or a yeast.

The IFO numbers given above denote the deposit numbers at the Institute for Fermentation, Osaka (IFO) and the FERM P or FERM BP numbers denote the deposit numbers at the Fermentation Research Institute (FRI), Agency of Industrial Science and Technology, Ministry of International Trade and Industry;

the same shall apply hereinafter.

Typical mycological properties of Amycolatopsis sp. TS-1-60 are shown below.

(a) Morphology

After shaking at 28°C for 24 to 48 hours in a nutrient liquid medium [e.g. Trypticase Soy Broth, (Becton Dickinson)], the substrate mycelia divide into small fragments resembling rod bacteria or branched short lengths of hyphae.

The substrate mycelium on agar medium shows zigzag forms. The aerial mycelium usually exhibits straight or flexuous (*Rectus Flexibilis*).

Acoremium is observed on agar media ISP-2 and ISP-7. The spore shows a cylindrical form (0.3 to 0.5X 0.6 to 2.3  $\mu$ m) with smooth surfaces.

(b) Cultural characteristics

Cultural characteristics of TS-1-60 strain on various media are shown in Table 2. Colors represented in Table 1 are determined by comparing them with color chips of the "Color Harmony Manual", 4th edition, Container Corporation of America.

Table 1      Cultural characteristics of strain TS-1-60 on Various Media

Medium	Growth	Color of Aerial Mycelium	Color of substrate mycelium (reverse Color)	Soluble pigment
Sucrose nitrate agar	Poor	Scant, white	Lt Ivory(2ca)	None
Glucose-asparagine agar	Abundant, flat	Moderate, white	Lt Ivory(2ca) to Pearl Pink(3ca)	None
Glycerol-asparagine agar	Abundant, wrinkled	Abundant, cottony white	Lt Wheat(2ea)	None
Inorganic salts-starch agar	Poor	Scant, white	Colorless to Lt Ivory(2ca)	None
(ISP medium 4)				
Tyrosine agar	Abundant	Abundant, cottony white	Lt Wheat(2ea)	Faint yellow
(ISP medium 7)				
Nutrient agar	Moderate	None	Lt Ivory(2ca)	None
Yeast extract-malt extract agar	Abundant, wrinkled, raised up with crack	Moderate, white	Honey Gold(2ic)	Faint yellow
(ISP medium 2)				
Oatmeal agar	Moderate, flat	Poor, white	Lt Ivory(2ca) to Lt Wheat(2ea)	None
(ISP medium 3)				

(c) Physiological characteristics

Formation of melanin pigment	Negative
Coagulation of milk	Negative
Peptonization of milk	Negative
Liquefaction of gelatin	Negative
Hydrolysis of starch	Negative
NaCl tolerance (%)	5< >7
Temperature range for growth (°C)	13 - 37
Optimum temperature for growth (°C)	20 - 30
Temperature for no growth (°C)	40

## (d) Utilization of carbon source

Positive: L-Arabinose, D-Xylose, D-Glucose, D-Fructose, Inositol, D-Mannitol

Negative: Sucrose, L-Rhamnose, Raffinose

As the plasmid suited for insertion of said DNA therein, there may be mentioned, among others, *Escherichia coli*-derived pBR322 [Gene, 2, 95 (1977)], pBR325 [Gene, 4, 121 (1978)] and pUC13 [Gene, 19, 259, (1982)]. Any other plasmid may also be used if it can be replicated and maintained in the host. As the phage vector for DNA insertion therein, there may be mentioned, for instance,  $\lambda$ gt11 [Young, R. and Davis, R., Proc. Natl. Acad. Sci. U.S.A., 80, 1194 (1983)]. Other phage vectors may be used as well if they can multiply in the host.

As the method of inserting said DNA into a plasmid, there may be mentioned, for instance, the method described in Maniatis, T. et al., Molecular Cloning, Cold Spring Harbor Laboratory, page 239 (1982). Suited for DNA insertion into a phage vector is the method of Hyunh, T. V. et al., DNA Cloning, a practical approach, 1, 49 (1985), for instance. The recombinant plasmid or phage vector thus obtained is introduced into an appropriate host, for example *Escherichia coli*.

As examples of said *Escherichia coli*, there may be mentioned, among others, *Escherichia coli* K12, DH1 [Proc. Natl. Acad. Sci. U.S.A., 60, 160 (1968)], JM103 [Nucl. Acids Res., 9, 309 (1981)], JA221 [J. Mol. Biol., 120, 517 (1978)], HB101 [J. Mol. Biol., 41, 459 (1969)] and C600 [Genetics, 39, 440 (1954)].

As the method of transforming a host with the plasmid, there may be mentioned, for instance, the calcium chloride method described in Maniatis, T. et al., Molecular Cloning, Cold Spring Harbor Laboratory, page 239 (1982), or the calcium chloride/rubidium chloride method. The phage vector can be introduced into grown *Escherichia coli*, for instance, by using the *in vitro* packaging technique.

An acylamino acid racemase DNA-containing actinomycetous DNA library can be obtained by the above-mentioned method or the like.

The acylamino acid racemase DNA can be cloned from among the actinomycetous DNA library, for example by the method of Huynh et al. [DNA Cloning, a practical approach, 1, 49 (1985)] using the phage vector  $\lambda$ gt11 and an acylamino acid racemase antibody or the colony hybridization or plaque hybridization method [Maniatis, T. et al., Molecular Cloning, Cold Spring Harbor Laboratory, (1982)] using, as a probe, an oligonucleotide chemically synthesized based on the amino acid sequence of *Amycolatopsis* sp. TS-1-60-derived acylamino acid racemase.

The thus-cloned acylamino acid racemase DNA can be subcloned, if necessary or where appropriate, into a plasmid, for example pBR322, pUC12, pUC13, pUC18, pUC19, pUC118 or pUC119.

The thus-obtained DNA is sequenced, for example by the Maxam-Gilbert method [Maxam, A. M. and Gilbert, W., Proc. Natl. Acad. Sci. U.S.A., 74, 560 (1977)] or the dideoxy method [Messing, J. et al., Nucl. Acids Res., 9, 309 (1981)] or the deaza method [Mizusawa, S. et al., Nucl. Acids Res., 14, 1319 (1986)] and the base sequence is compared with the known amino acid sequence to thereby confirm the existence of the acylamino acid racemase DNA. In cases where all the region encoding acylamino acid racemase is not covered, colony hybridization is carried out using the DNA fragment as a probe for recloning of the acylamino acid racemase to thereby fill up the missing region.

In the above manner, a DNA coding for acylamino acid racemase is obtained.

As a typical example of the DNA according to the invention which encodes acylamino acid racemase, there may be mentioned the acylamino acid racemase-encoding DNA obtained in Example 1 to be described later herein. Its restriction enzyme cleavage map is shown in Fig. 2.

The DNA encoding acylamino acid racemase as cloned in the above manner can be used either as such where appropriate or, if desired, after restriction enzyme digestion or site-directed mutagenesis [Methods in Enzymology, 100, 468 (1983)], for instance, for improving the plasmid.

The above-mentioned cloned DNA encoding N-acylamino acid racemase can be expressed in large amounts in *Escherichia coli*, for instance, using a promoter such as the lac promoter, tac promoter [de

Boyer, H. A., Camstock, L. J., Vasser, M., Proc. Natl. Acad. Sci. U.S.A., 80, 21 (1980)], T7 promoter [Tabor, S., Richardson, C. C., Proc. Natl. Acad. Sci. U.S.A., 82, 1074 (1985)].

A microorganism (e.g. actinomycete, *Escherichia coli*, yeast) transformed with the above-mentioned cloned DNA is cultivated, whereby acylamino acid racemase is produced in the culture medium and can be recovered therefrom.

It is also possible to improve some property or properties of acylamino acid racemase itself (e.g. enzyme activity, stability) by the above-mentioned methods.

As the host to be used for the expression, there may be mentioned *Streptomyces lividans* TK64 and *Escherichia coli* HB101, for instance. Also usable are other actinomycetes, other *Escherichia coli* strains, *Bacillus subtilis* and, further, yeasts.

The transformation of actinomycetes is per se known and is carried out by the method of Hopwood, D. A. et al. as described in Genetic Manipulation of *Streptomyces*, A Laboratory Manual).

The transformation of *Escherichia coli* is also known and can be performed by the calcium chloride method described in Maniatis, T. et al., Molecular Cloning, Cold Spring Harbor Laboratory, page 239, 1982, or by the calcium chloride/rubidium chloride method.

As a typical example of such transformant according to the invention, there may be mentioned *Escherichia coli* ER-4 (IFO 15083; FERM BP-3091).

It is possible, of course, to produce various transformants with ease in the same manner as described herein by appropriately selecting receptor organisms.

The thus-obtained transformants are cultivated by a per se known method. In cultivating actinomycetes, the seed medium is as follows: 3% glycerol, 0.5% polypeptone, 0.3% meat extract, 0.05% N-acetyl-DL-methionine, pH 7.2; and the production medium is as follows: 1.7% pancreatin-treated casein, 0.3% papain-treated defatted soybean flour, 0.5% sodium chloride, 0.25% dipotassium hydrogen phosphate, 1% glucose, 0.05% N-acetyl-DL-methionine, pH 7.0. The cultivation is carried out generally at 15-40°C, preferably at 24-30°C, for 10-96 hours, preferably for 24-72 hours. If necessary, aeration and/or agitation may be made. In cultivating *Escherichia coli*, LB medium (1% polypeptone, 0.5% yeast extract, 0.5% sodium chloride) is used as the medium. The cultivation is carried out generally at 15-40°C, preferably at 24-37°C, for 10-96 hours, preferably for 24-48 hours. If necessary, aeration and/or agitation may be made.

After completion of the cultivation, the cells and the supernatant are separated from each other. The acylamino acid racemase remaining in the cells is extracted by a cell disruption method commonly used in this field of art, for example sonication, disruption by means of a French press, mechanical disruption by grinding or the like, or treatment with lysozyme. The acylamino acid racemase contained in the thus-obtained extract is purified by subjecting said extract to a usual method of protein purification, for example salting out, isoelectric precipitation, gel filtration, ionexchange chromatography, hydrophobic chromatography, or high-performance liquid chromatography, whereby the desired acylamino acid racemase can be obtained.

The activity of the acylamino acid racemase obtained in the above manner is measured by the known method (Japanese Kokai Tokyo Koho JP 01-137973). Thus, 50-100 µl of an enzyme solution is added to a reaction medium comprising 25 mM N-acetyl-DL-methionine, 2 mM cobalt chloride, 2 U L-acylase and 50 mM Tris-hydrochloride buffer (pH 7.5) and the reaction is allowed to occur at 30°C for 5 minutes and then stopped by boiling for 3 minutes. One unit (U) of enzyme is defined as an amount of enzyme causing formation of 1 µmol of L-methionine per minute.

The abbreviations and symbols used herein for bases, amino acids, etc. are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature or commonly used in the relevant fields. Examples are shown below. As for those amino acids which may occur in optical isomer forms, the abbreviations used, unless otherwise specified, denote the L isomers.

DNA	Deoxyribonucleic acid
A	Adenine
C	Cytosine
G	Guanine
T	Thymine
Ala	Alanine
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
Cys	Cysteine
Gln	Glutamine
Glu	Glutamic acid

Gly	Glycine
His	Histidine
Ile	Isoleucine
Leu	Leucine
5 Lys	Lysine
Met	Methionine
Phe	Phenylalanine
Pro	Proline
Ser	Serine
10 Thr	Threonine
Trp	Tryptophan
Tyr	Tyrosine
Val	Valine

## 15 Examples

The following examples are further illustrative of the present invention but are by no means limitative of the scope thereof.

### 20 Example 1

#### Isolation of chromosomal DNA of Amycolatopsis sp. TS-1-60

Amycolatopsis sp. TS-1-60 was shake-cultured in a 200-ml erlenmeyer flask containing 20 ml of Trypticase Soy Broth (Becton Dickinson) at 28° C for 42 hours. The wet cells (4 g) obtained were suspended in 20 ml of buffer A [0.3 M sucrose, 25 mM EDTA, 25 mM Tris-HCl buffer (pH 8)] and, after addition of egg white lysozyme to a concentration of 2 mg/ml, the suspension was shaken gently at 37° C for 1 hour. Then, 8 ml of 2% SDS was added to the suspension, followed by two or three gentle swirlings. Thereto were added 5 ml of chloroform and 5 ml of buffer A-saturated phenol, and the whole mixture was centrifuged (3,000 rpm, 20 minutes) for DNA extraction. After three repetitions of this phenol extraction, one tenth volume of 3 M potassium acetate and 2 volumes of ice-cooled ethanol were added to the DNA solution for DNA precipitation. The precipitate DNA was collected by centrifugation, washed with 70% ethanol, dried in vacuo, and stored.

### Example 2

#### Cloning of an acylamino acid racemase-encoding gene

##### (A) Preparation of a gene library

##### a) Partial digestion with the restriction enzyme HaeIII

The chromosomal DNA of Amycolatopsis sp. TS-1-60 as isolated in Example 1 was partially digested with the restriction enzyme HaeIII (Takara Shuzo). Thus, the reaction was allowed to proceed at 37° C for 2 minutes in a total volume of 50  $\mu$ l of a reaction system composed of 10 mM Tris-HCl buffer (pH 7.5), 7 mM magnesium chloride, 60 mM sodium chloride, 7 mM 2-mercaptoethanol, 5  $\mu$ g of the donor DNA and 10 units of HaeIII. The reaction mixture was deproteinized by phenol-chloroform treatment and the digest DNA was recovered by addition of 2 volumes of ethanol.

##### b) Methylase reaction

The DNA fragment partially digested with HaeIII was methylated on the adenosine moiety in the EcoRI site. Thus, 5  $\mu$ g of the partial digest DNA fragment was treated in a total volume of 20  $\mu$ l of a reaction medium composed of 100 mM Tris-HCl buffer (pH 8), 2 mM dithiothreitol, 10 mM EDTA, 80  $\mu$ M S-adenosylmethionine and 40 units of methylase (Takara Shuzo) at 37° C for 60 minutes. The methylated DNA fragment was recovered in the same manner as mentioned above.

##### c) Linker ligation

An EcoRI linker was joined to the terminus of the methylated DNA fragment. The EcoRI linker used was d (pGGAATTCC) (Takara Shuzo). For the linker joining, a ligation kit (Takara Shuzo) was used. The reaction was carried out at 16° C for 16 hours using a total of 20  $\mu$ l of a mixture composed of 5  $\mu$ g of the DNA, 2  $\mu$ l (2  $\mu$ g) of the EcoRI linker, 16  $\mu$ l of solution A and 2  $\mu$ l of solution B. The reaction mixture was treated with chloroform/phenol and the product DNA was then recovered by ethanol precipitation followed by drying.

##### d) Digestion with the restriction enzyme EcoRI

5  $\mu$ g of the DNA fragment obtained in c) was digested with the restriction enzyme *EcoRI* at 37°C for 3 hours. The resultant DNA fragments were electrophoresed and DNA fragments 1 to 3 kbp in size were recovered, subjected to phenol-chloroform treatment and ethanol precipitation and, after drying in vacuo, dissolved in 20  $\mu$ l of TNE buffer (10 mM Tris-HCl, 1 mM EDTA, 300 mM NaCl, pH 8.0).

5 e) Ligation with vector ( $\lambda$ gt11) and packaging

$\lambda$ gt11 (Stratagene Cloning Systems, U.S.A.) was treated with *EcoRI* and with phosphatase and ligated with the DNA fragments (1-3 kbp) and the ligation reaction mixture was packaged using Gigapack gold (Stratagene Cloning Systems, U.S.A.).

10 (B) Preparation of probes

Acylamino acid racemase (purified protein, 4 mg) produced by *Amycolatopsis* sp. TS-1-60 was cleaved into several peptides by treatment with lysyl endopeptidase. The peptides were purified by reversed-phase chromatography and the amino acid sequence of a peptide named peak 2 was determined. The sequence was Lys Leu Gly Ala Val Gln Ile Val Asn Ile Lys Pro Gly Arg Val Gly Gly Tyr. The following oligonucleotide deduced from the underlined peptide portion was synthesized:

15 5'-GAGATCCTR<sup>4</sup>AACATCAAGCC-3' (R<sup>4</sup> being G or C). This oligonucleotide was labeled with <sup>32</sup>P at the 5' end by the conventional method for its use as a probe.

(c) Preparation of an antibody against acylamino acid racemase

Rabbits were challenged with about 1 mg of the purified acylamino acid racemase protein obtained from *Amycolatopsis* sp. TS-1-60 in four divided doses (400, 200, 200 and 200  $\mu$ g). As a result, a polyclonal antibody-containing serum was obtained which had an antibody titer of 16 as determined by the Ouchterlony method.

(D) Screening by immunoassay

25 *Escherichia coli* Y1090 was transfected with the  $\lambda$ gt11 DNA library derived from *Amycolatopsis* sp. TS-1-60 and 13 positive plaques were obtained from among about 200,000 plaques. They were purified and proliferated, the DNAs were extracted therefrom and cleaved with *EcoRI*, and the digests were subjected to electrophoresis. The DNA fragments separated by electrophoresis were labeled with P<sup>32</sup> and subjected to Southern hybridization using the probe mentioned above. Of the 13 recombinant phages, two phages,  $\lambda$ -8 and  $\lambda$ -9, hybridized with the probe. Then, using part of the *EcoRI* fragments of  $\lambda$ -9, a probe of about 600 bp was prepared. Using this probe, plaque hybridization was again performed with about 50,000 plaques obtained from the  $\lambda$ gt11 DNA library. As a result,  $\lambda$ -44 was obtained and, upon minute investigation, this was found to contain the whole DNA encoding acylamino acid racemase.

Example 3

35 Base sequence determination (sequencing)

An *EcoRI* fragment (1.2 kbp) isolated from  $\lambda$ -44 was inserted into the *Escherichia coli* phages M13mp8 and M13mp9 by the method of Messing et al. [Nucl. Acids Res., 9, 309 (1981)] and sequenced by the deaza method [Mizusawa, S. et al., Nucl. Acids Res., 14, 1319 (1986)]. The results is shown in Fig. 1. Part of the amino acid sequence deduced from this base sequence was in agreement with that part of the amino acid sequence of acylamino acid racemase purified from *Amycolatopsis* sp. TS-1-60. It was thus found that  $\lambda$ -44 contained a gene encoding acylamino acid racemase.

Example 4

45 Production of acylamino acid racemase using a transformant of an actinomycete

*Streptomyces lividans* TK64/pRA32 was used. This strain was produced by extracting the acylamino acid racemase gene from the whole DNA of *Amycolatopsis* sp. TS-1-60, inserting said gene into pIJ702, a plasmid for use in actinomycetes, and transforming *Streptomyces lividans* TK64 with the resulting recombinant plasmid. Said strain was cultured on a yeast extract-malt extract agar slant medium (ISP2 medium) at 28°C for 7 days. One loopful of the spores thus formed was inoculated into 20 ml of sterilized seed medium (3% glycerol, 0.5% polypeptone, 0.3% meat extract, 0.05% N-acetyl-DL-methionine, pH 7.2) in each 200-ml erlenmeyer flask and cultured on a rotary shaker (200 rpm) at 28°C for 48 hours. The culture was distributed in 8-ml portions into 15-ml vials and stored frozen at -80°C (frozen stock culture). The first seed was prepared by inoculating the same seed medium as mentioned above with 1 ml of the frozen stock culture and conducting cultivation under the same conditions as mentioned above. The second seed was prepared by inoculating a sterilized 500-ml portion of the seed medium in each 2-liter Sakaguchi flask with 20 ml of the first seed culture and carrying out cultivation on a reciprocal shaker (78 spm) at 28°C for 72 hours. For enzyme production, 100 liters of a production medium (1.7% pancreatin-treated casein, 0.3%

papain-treated defatted soybean flour, 0.5% sodium chloride, 0.25% dipotassium hydrogen phosphate, 1% glucose, 0.05% N-acetyl-DL-methionine, pH 7.0) placed and sterilized in each 200-liter fermentor was inoculated with 4 liters of the second seed culture and cultivation was performed at 28 °C for 42 hours with aeration (80 vvm) and agitation (160 rpm). As a control, the DNA donor *Amycolatopsis* sp. TS-1-60 was cultivated in the same manner. Comparison from the enzyme production viewpoint revealed that the transformant showed higher productivity.

#### Example 5

Expression of the acylamino acid racemase gene in *Escherichia coli* using the lac promoter  
A *Sma*I-*Xho*I fragment containing the structural gene for acylamino acid racemase was separated from the recombinant phage  $\lambda$ -44. This fragment was inserted into the *Escherichia coli* plasmid pUC118 at the multicloning site (*Sma*I-*Sall* site) thereof in the same orientation as that of the lac promoter to give a plasmid, pRA4 (Fig. 3). This plasmid pRA4 was used to transform *Escherichia coli* JM105.

The resultant transformant strain ER-4 (IFO 15083; FERM BP-3091) was picked up with a bamboo stick for inoculation of 4 ml of LB medium (1% polypeptone, 0.5% yeast extract, 0.5% sodium chloride) containing ampicillin (50  $\mu$ g/ml) and shake culture was carried out at 37 °C for 16 hours. A 0.3-ml portion of the culture was inoculated into 30 ml of LB medium containing ampicillin (50  $\mu$ g/ml) and shake culture was performed at 37 °C for 3 hours. Then, 3 ml of 0.1 M IPTG (isopropyl  $\beta$ -D-thiogalactopyrano-side) was added (final concentration 1 mM) and cultivation was continued further for 4 hours. Cells were collected (3,000 rpm, 10 minutes), suspended in 5 ml of Tris-hydrochloride buffer (50 mM, pH 7.5) and sonicated (4 minutes). The resultant cell disruption product was subjected to centrifugation (15,000 rpm, 10 minutes) and the supernatant was subjected to acylamino acid racemase activity determination by the known method. Acylamino acid racemase activity, which was not observed with the host *Escherichia coli* JM105, could be confirmed with the transformant ER-4. The enzyme productivity was 64 U/liter (medium) and about 3 times as compared with the DNA donor *Amycolatopsis* sp. TS-1-60.

#### Example 6

Expression of the acylamino acid racemase gene in *Escherichia coli* using the tac promoter  
A *Sma*I-*Hind*III fragment containing the structural gene for acylamino acid racemase was excised from the lac expression plasmid pRA4 of Example 5. This fragment was inserted into the tac promoter-containing expression plasmid vector pKK223-3 [Brosius, J and Holly, A., Proc. Natl. Acad. Sci. U.S.A., 81, 6929 (1984)] at the *Sma*I-*Hind*III site thereof to give a plasmid, pRA7 (Fig. 4). This plasmid pRA7 was used to transform *Escherichia coli* JM105.

The resultant transformant ER7 was cultivated by the method described in Example 5 and the acylamino acid racemase activity was measured by the known method, whereby said enzyme activity was observed. The enzyme productivity was 34 U/liter (medium), which was about 1.5 times higher than that found with the DNA donor *Amycolatopsis* sp. TS-1-60.

#### Example 7 Initiation codon conversion from GTG to ATG

An initiation codon-containing *Eco*RI fragment was separated and recovered from the recombinant phage  $\lambda$ -44 and inserted into the plasmid vector pUC118 at the *Eco*RI site thereof. The resultant recombinant plasmid was used to transform *Escherichia coli* MV1184 and a single-stranded DNA was prepared from a transformant obtained by the method of Vieira et al. [Methods in Enzymology, 100, 3 (1987)]. Site-directed mutagenesis was performed using a mutation point-containing synthetic oligonucleotide 5'-GAGGAGCAATGAACTC-3' in in vitro Mutagenesis System, version 2.0 (Amersham). A mutation point-containing *Sac*I fragment was separated and recovered from the resultant mutant plasmid pMA1 and joined to the expression plasmid pRA4 treated in advance with *Sac*I, to give a lac promoter expression plasmid, pRA4A, in which the initiation codon had been converted to ATG (Fig. 5). Similarly, a mutation point-containing *Sma*I/*Sac*I fragment was separated and recovered from the mutant plasmid pMA1 and joined to the expression plasmid pRA7 treated in advance with *Sma*I/*Sac*I, to give a tac promoter expression plasmid, pRA7A, in which the initiation codon had been converted to ATG (Fig. 6). These mutant expression plasmids were used to transform *Escherichia coli* JM105, respectively giving transformants, ER4A (JM105/pRA4A) and ER7A (JM105/pRA7A; IFO 15131, FERM BP-3272). These transformants were cultivated by the method of Example 5 for 9 hours following addition of IPTG and acylamino acid racemase activity measurements were made by the per se known method. The enzyme productivities were 740 U/liter

(medium) for the strain ER4A and 198 U/liter (medium) for the strain ER7A, being about 37 times and about 10 times higher, respectively, as compared with the DNA donor Amycolatopsis sp. TS-1-60. Example 8 Expression of acylamino acid racemase gene in

Escherichia coli using T7 promoter

- 5 For subcloning the acylamino acid racemase gene in the T7 expression plasmid pET-3c [Gene, 56, 125 (1987)] at the NdeI/BamHI site thereof, an NdeI site (CATATG) was introduced into the initiation codon region and a BglII site (AGATCT) into the termination codon region using the technique of site-directed mutagenesis.

A) NdeI site introduction

- 10 Escherichia coli MV1184 was transformed with the expression plasmid pRA4 and a single-stranded DNA was prepared from one of the thus-obtained transformants using the method of Vieira et al. Using this single-stranded DNA and an NdeI site (CATATG)-containing synthetic oligonucleotide (5'-GAGTTTCATATGCTCCTCC-3'), site-directed mutagenesis [in, vitro Mutagenesis System, version 2.0 (Amersham)] was carried out to give a plasmid, pRA4N, having an NdeI site in the initiation codon region (Fig. 7).

B) BglII site introduction

- 20 An NdeI site-containing SmaI/HindIII fragment was separated from the plasmid pRA4N and joined to the plasmid pUC119 treated in advance with SmaI and HindIII. The resultant recombinant plasmid was used to transform Escherichia coli MV1184. A single-stranded DNA was prepared from one of the thus-obtained transformants by the method of Vieira et al. Using this single-stranded DNA and a BglII site (AGATCT)-containing synthetic oligonucleotide (5'-GAGGTAGATCTGGTCGGAT-3'), site-directed mutagenesis was performed in the same manner as in A) to give a plasmid, pRA4NB, having a BglII site in the termination codon region (Fig. 8).

C) Expression

- 25 An NdeI/BglII fragment containing the enzyme gene in question was separated and recovered from the plasmid pRA4NB and inserted into the plasmid pET-3c between the NdeI site and BamHI site thereof to give an acylamino acid racemase expression plasmid, pET-3cN (Fig. 9). This plasmid was used to transform Escherichia coli MM294 (DE3), whereby a transformant, MR-1 (IFO 15132, FERM BP-3273), was obtained. This transformant was cultivated by the method described in Example 7 and the acylamino acid racemase activity as measured by the per se known method. The enzyme productivity was 1,795 U/liter (medium). This was about 90 times higher as compared with the DNA donor Amycolatopsis sp. TS-1-60.

D) Tank culture (20 liters) of transformant MR-1

- 35 LB medium containing ampicillin (50 µg/ml) was distributed in 400-ml portions into one-liter erlenmeyer flasks and sterilized. The medium in each flask was inoculated with one loopful of cells of the strain MR-1. Shake culture (250 rpm) was carried out at 37°C for 20 hours. One liter of the culture fluid thus obtained was inoculated into 20 liters of M9 medium [Maniatis, T. et al., Molecular Cloning, Cold Spring Harbor Laboratory (1982)] supplemented with polypeptone (1.5%) and cultured at 28°C for 34 hours with aeration (100 vvm) and stirring (450 rpm). At 8 hours after initiation of cultivation, IPTG was added to a final concentration of 0.01 mM and cultivation was continued with continuous addition (150 ml/hour) of a mixed solution of 8% glucose and 2% polypeptone. After completion of the cultivation, the culture was centrifuged (1,000 rpm, 20 minutes) to give 1,320 g of wet cells. The acylamino acid racemase activity was measured by the per se known method. The enzyme productivity was 22,300 U/liter (medium). This was about 1,100 times higher as compared with the DNA donor Amycolatopsis sp. TS-1-60.

Example 9 Enzyme purification from Escherichia coli transformant

- 50 LB medium containing ampicillin (50 µg/ml) was distributed in 40-ml portions into 200-ml erlenmeyer flasks and sterilized. The medium in each flask was inoculated with one loopful of cells of the strain ER4A and shake culture (300 rpm) was conducted at 37°C for 16 hours. The culture fluid thus obtained was transferred in 4-ml portions into one-liter erlenmeyer flasks each containing 400 ml of sterilized LB medium and shake culture (250 rpm) was carried out at 30°C for 30 hours. At about 6 hours after initiation of cultivation, 4 ml of IPTG (0.1 M) was added to each flask (final concentration 1 mM). Eight liters of the culture fluid was centrifuged (10,000 rpm, 20 minutes) to give 141 g of wet cells.

These cells were suspended in Tris-hydrochloride buffer (50 mM, pH 7.5) to make 500 ml and then disrupted by sonication (5 minutes x 3). The cell disruption product fluid was made 1 liter by addition of the same buffer and magnesium sulfate (final concentration 10 mM), heat-treated (60°C, 30 minutes) and then

centrifuged (10,000 rpm, 20 minutes) to give 940 ml of a supernatant. To this supernatant was added 107.2 g (for 20% saturation) of ammonium sulfate. The resultant solution was allowed to stand at 0 °C for 2 hours and then centrifuged (10,000 rpm, 30 minutes), and the supernatant thus obtained was applied to a BUTYL-Toyopearl column (BUTYL-Toyopearl 650M, Tosoh, 4.5 x 30 cm) equilibrated in advance with Trishydrochloride buffer containing 20% saturated ammonium sulfate. The column was washed with 2 liters of the same buffer containing 20% saturated ammonium sulfate, followed by elution with the same buffer while the ammonium sulfate solution was varied from 20% saturation to 0% saturation. The eluate was fractionated in 20-ml portions. The acylamino acid racemase activity was observed in the 34th to 50th fractions. The active fractions were combined, dialyzed against the same buffer and applied to a DEAE-Toyopearl column (DEAE-Toyopearl 650M, Tosoh, 4.1 x 16 cm) equilibrated in advance with the same buffer. The column was washed with 1 liter of the same buffer. Elution was carried out with 1 liter of the same buffer containing sodium chloride whose concentration was varied from 0 to 0.5 M. The eluate was fractionated in 10-ml portions. The acylamino acid racemase activity was observed in the 48th to 53rd fractions. The above procedure gave 1,570 U of acylamino acid racemase showing a substantially single band in SDS-polyacrylamide gel electrophoresis. The results of purification of this enzyme from the transformant ER4A are shown below.

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purity (fold)	Yield (%)
Cell disruption	18000	3180	0.17	1.0	100
Heat treatment	7123	3256	0.46	2.7	100
BUTYL-Toyopearl	249	1620	6.50	38.2	51
DEAE-Toyopearl	198	1573	13.5	79.4	49

#### Claims

1. A DNA fragment containing a gene encoding acylamino acid racemase.
2. A DNA fragment according to claim 1, wherein the gene is derived from a microorganism.
3. A DNA fragment according to claim 2, wherein the microorganism is an actinomycete.
4. A DNA fragment according to claim 2, wherein the gene hybridizes with a DNA whose base sequence is represented by for formula:  
5' -GAGATCCTR<sup>4</sup> AACATCAAGCC-3'  
wherein R<sup>4</sup> represent either G or C.
5. A DNA fragment according to claim 2, wherein the gene has a base sequence of No. 62 to No. 1189 shown in Figure 1.
6. A DNA fragment according to claim 2, wherein the gene has a base sequence of No. 1 to No. 1400 shown in Figure 1.
7. A vector having a DNA fragment containing a gene encoding acylamino acid racemase.
8. A plasmid having a DNA fragment containing a gene encoding acylamino acid racemase.
9. A transformant carrying a vector having a DNA fragment containing a gene encoding acylamino acid racemase.
10. A transformant carrying a plasmid having a DNA fragment containing a gene encoding acylamino acid racemase.
11. A method for producing acylamino acid racemase which comprises cultivating a transformant carrying a plasmid having a DNA fragment containing a gene encoding acylamino acid racemase in a medium to produce and accumulate acylamino acid racemase in the culture and then recovering said racemase.

Fig. 1 (1)

GAATTCCTCCG GGTGACCGGC TCGACCGAG CCGGCTTTTA CGTGATCTCC AAGGAGGAGC 60  
 A GTG AAA CTC AGC GGT GTG GAA CTG CGC CGG GTG CAG ATG CCG CTC GTC 109  
 Met Lys Leu Ser Gly Val Glu Leu Arg Arg Val Gln Met Pro Leu Val  
 1 5 10 15  
 GCC CCG TTC CGG ACT TCG TTC GGC ACC CAG TCG GTC CGC GAG CTC TTG 157  
 Ala Pro Phe Arg Thr Ser Phe Gly Thr Gln Ser Val Arg Glu Leu Leu  
 20 25 30  
 CTG CTG CGC GCG GTC ACG CCG GCC GGC GAG GGC TGG GGC GAA TGC GTG 205  
 Leu Leu Arg Ala Val Thr Pro Ala Gly Glu Gly Trp Gly Glu Cys Val  
 35 40 45  
 ACG ATG GCC GGT CCG CTG TAC TCG TCG GAG TAC AAC GAC GGC GCG GAA 253  
 Thr Met Ala Gly Pro Leu Tyr Ser Ser Glu Tyr Asn Asp Gly Ala Glu  
 50 55 60  
 CAC CTG CTG CGG CAC TAC TTG ATC CCG GCG CTG CTG GCC GCG GAA GAC 301  
 His Val Leu Arg His Tyr Leu Ile Pro Ala Leu Leu Ala Ala Glu Asp  
 65 70 75 80  
 ATC ACC GCG GCG AAG GTG ACG CCG CTG CTG GCC AAG TTC AAG GGC CAC 349  
 Ile Thr Ala Ala Lys Val Thr Pro Leu Leu Ala Lys Phe Lys Gly His  
 85 90 95  
 CGG ATG GCC AAG GGC GCG CTG GAG ATG GCC GTG CTC GAC GCC GAA CTC 397  
 Arg Met Ala Lys Gly Ala Leu Glu Met Ala Val Leu Asp Ala Glu Leu  
 100 105 110  
 CGC GCG CAC GAG AGG TCG TTC GCC GCC GAA CTC GGA TCG GTG CGC GAT 445  
 Arg Ala His Glu Arg Ser Phe Ala Ala Glu Leu Gly Ser Val Arg Asp  
 115 120 125  
 TCT GTG CCG TGC GGC GTT TCG GTC GGG ATC ATG GAC ACC ATC CCG CAA 493  
 Ser Val Pro Cys Gly Val Ser Val Gly Ile Met Asp Thr Ile Pro Gln  
 130 135 140  
 CTG CTC GAC GTC GTG GGC GGA TAC CTC GAC GAG GGT TAC GTG CGG ATC 541  
 Leu Leu Asp Val Val Gly Gly Tyr Leu Asp Glu Gly Tyr Val Arg Ile  
 145 150 155 160

Fig. 1 (2)

AAG CTG AAG ATC GAA CCC GGC TGG GAC GTC GAG CCG GTG CGC GCG GTC	589
Lys Leu Lys Ile Glu Pro Gly Trp Asp Val Glu Pro Val Arg Ala Val	
165 170 175	
CGC GAG CGC TTC GGC GAC GAC GTG CTG CTG CAG GTC GAC GCG AAC ACC	637
Arg Glu Arg Phe Gly Asp Asp Val Leu Leu Gln Val Asp Ala Asn Thr	
180 185 190	
GCC TAC ACC CTC GGC GAC GCG CCG CAG CTG GCC CGG CTC GAC CCG TTC	685
Ala Tyr Thr Leu Gly Asp Ala Pro Gln Leu Ala Arg Leu Asp Pro Phe	
195 200 205	
GGC CTG CTG CTG ATC GAG CAG CCG CTG GAA GAG GAG GAC GTG CTC GGC	733
Gly Leu Leu Leu Ile Glu Gln Pro Leu Glu Glu Glu Asp Val Leu Gly	
210 215 220	
CAC GCC GAA CTG GCC CGC CGG ATC CAG ACA CCG ATC TGC CTC GAC GAG	781
His Ala Glu Leu Ala Arg Arg Ile Gln Thr Pro Ile Cys Leu Asp Glu	
225 230 235 240	
TCG ATC GTG TCG GCG CGC GCG GCG GCG GAC GCC ATC AAG CTG GGC GCG	829
Ser Ile Val Ser Ala Arg Ala Ala Ala Asp Ala Ile Lys Leu Gly Ala	
245 250 255	
GTC CAA ATC GTG AAC ATC AAA CCG GGC CGC GTC GGC GGG TAC CTG GAA	877
Val Gln Ile Val Asn Ile Lys Pro Gly Arg Val Gly Gly Tyr Leu Glu	
260 265 270	
GCG CGG CGG GTG CAC GAC GTG TGC GCG GCG CAC GGG ATC CCG GTG TGG	925
Ala Arg Arg Val His Asp Val Cys Ala Ala His Gly Ile Pro Val Trp	
275 280 285	
TGC GGC GGG ATG ATC GAG ACC GGC CTC GGC CGG GCG GCG AAC GTC GCG	973
Cys Gly Gly Met Ile Glu Thr Gly Leu Gly Arg Ala Ala Asn Val Ala	
290 295 300	
CTG GCC TCG CTG CCG AAC TTC ACC CTG CCC GGC GAC ACC TCG GCG TCG	1021
Leu Ala Ser Leu Pro Asn Phe Thr Leu Pro Gly Asp Thr Ser Ala Ser	
305 310 315 320	

Fig. 1 (3)

GAC CGG TTC TAC AAA ACC GAC ATC ACC GAG CCG TTC GTG CTC TCC GGC 1069  
 Asp Arg Phe Tyr Lys Thr Asp Ile Thr Glu Pro Phe Val Leu Ser Gly  
                   325                                  330                                  335  
 GGC CAC CTC CCG GTG GCC GAC CCG ACC GGG CCT CCG CGT GGC GCC GAT 1117  
 Gly His Leu Pro Val Ala Asp Arg Thr Gly Pro Arg Arg Gly Ala Asp  
                   340                                  345                                  350  
 TCC GGA GCT GCT GGA CGA GGT GAC CAC GGC AAA GGT GTG GAT CCG TTC 1165  
 Ser Gly Ala Ala Gly Arg Gly Asp His Gly Lys Gly Val Asp Arg Phe  
                   355                                  360                                  365  
 GTA GCC CGC TAC GAA TTC CCG AGG TAGATTTGGT CGGATCGGAC CAGCCGGTCCG 1219  
 Val Ala Arg Tyr Glu Phe Arg Arg  
                   370                                  375  
 CACGAGGCCG GATCTACCTT CGGGGGGTGC TGACACCGGT GCCGAGCAAA CCGCACACGA 1279  
 GTCTGGACGC GTCCTCGAAG CTCTCGGGGA CGTGCTCCTC GAGCCGGTCC CCGTCGGCGC 1339  
 GACACGGCGC GGCAGCTCGG CGGGGTGGTG ATTACGACC CGCACGACGA CGCGGAATT 1399  
 C 1400

Fig. 2

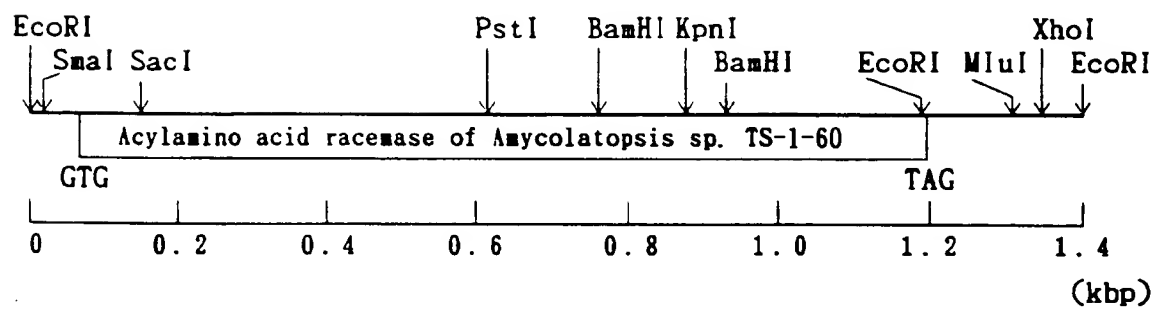


Fig. 3

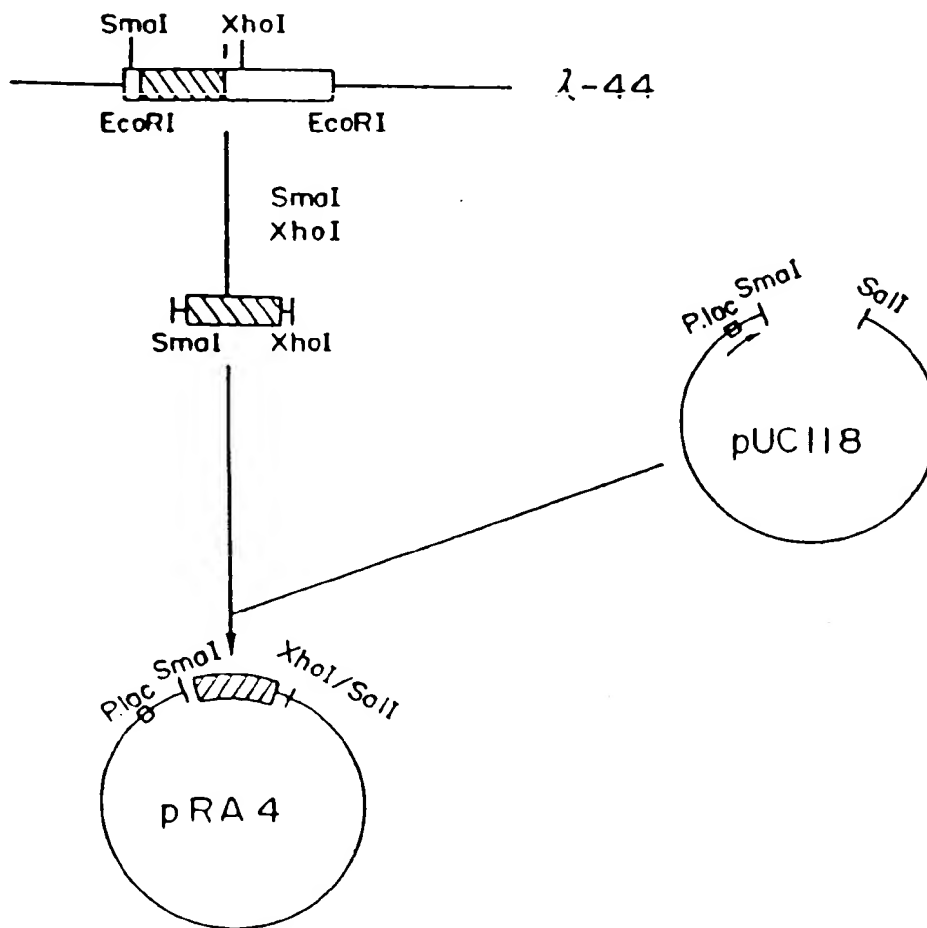


Fig. 4

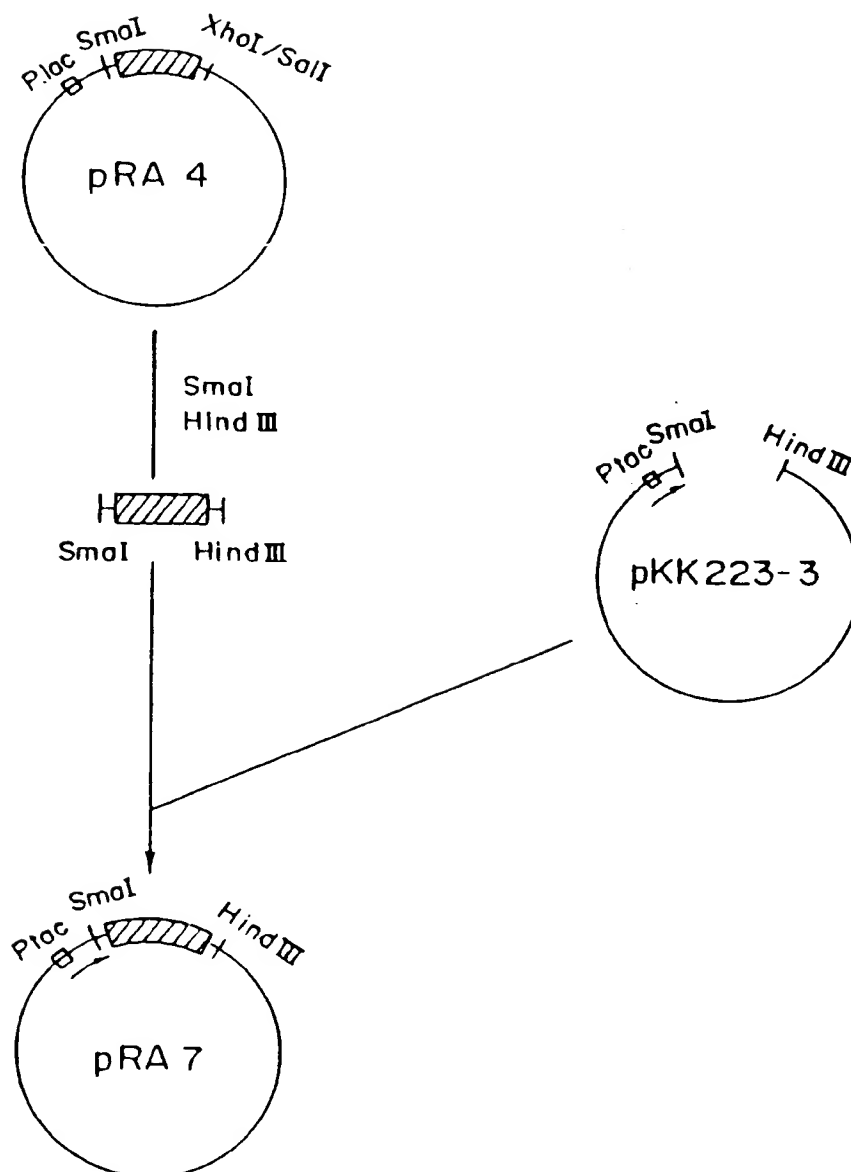


Fig. 5

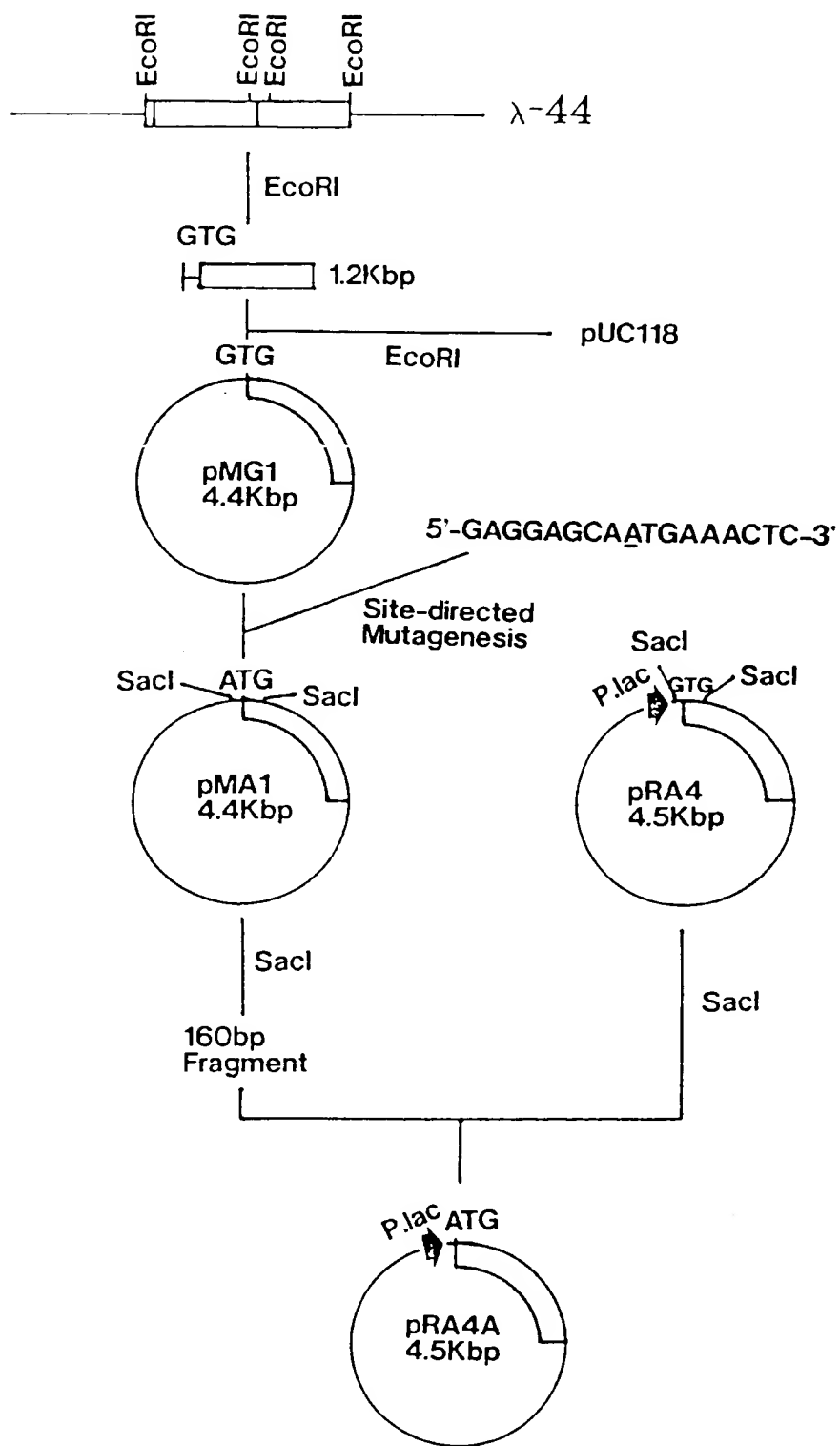


Fig. 6

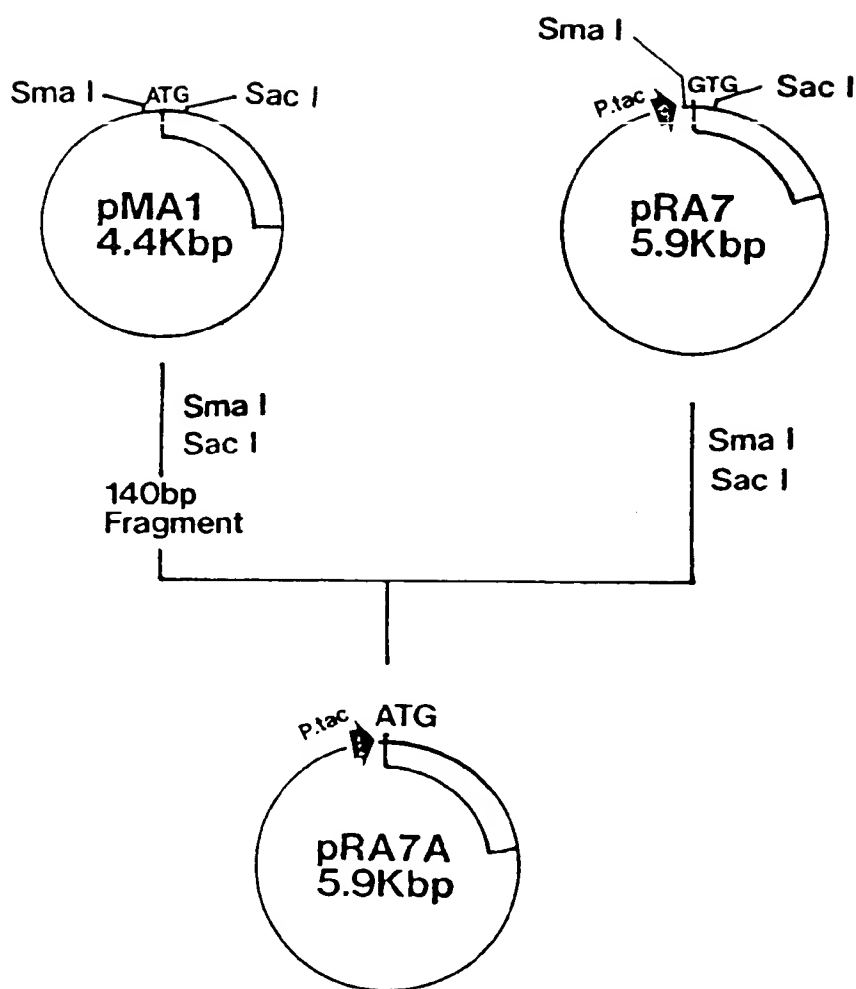


Fig. 7

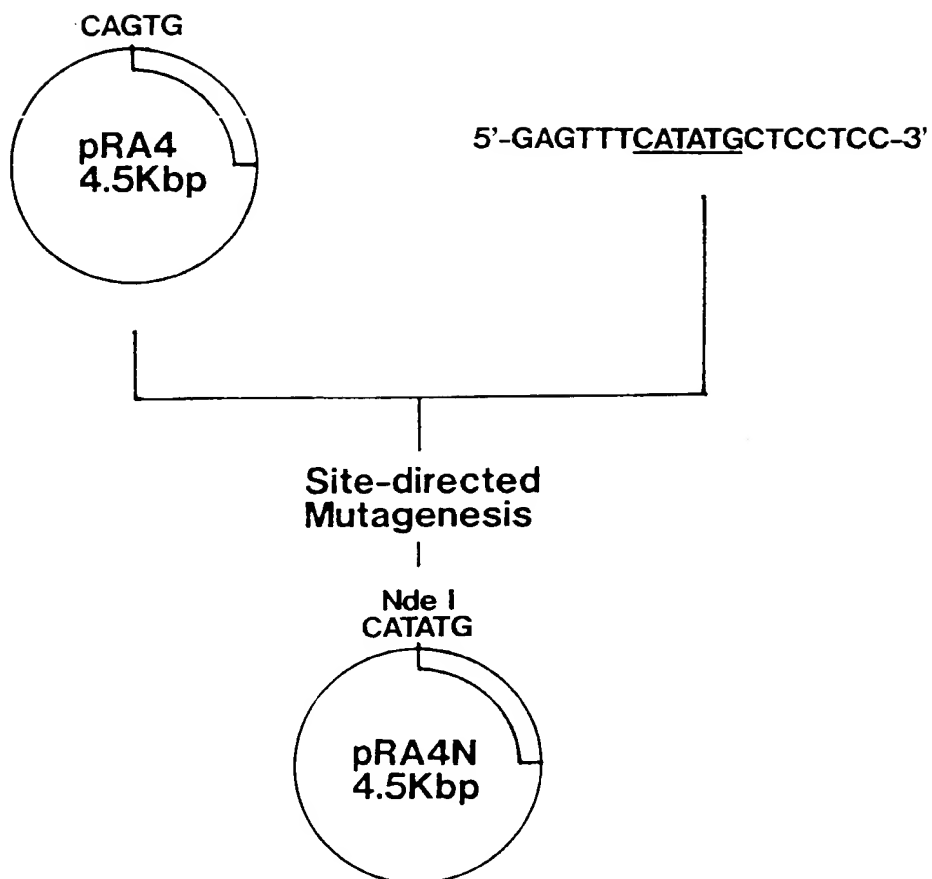


Fig. 8

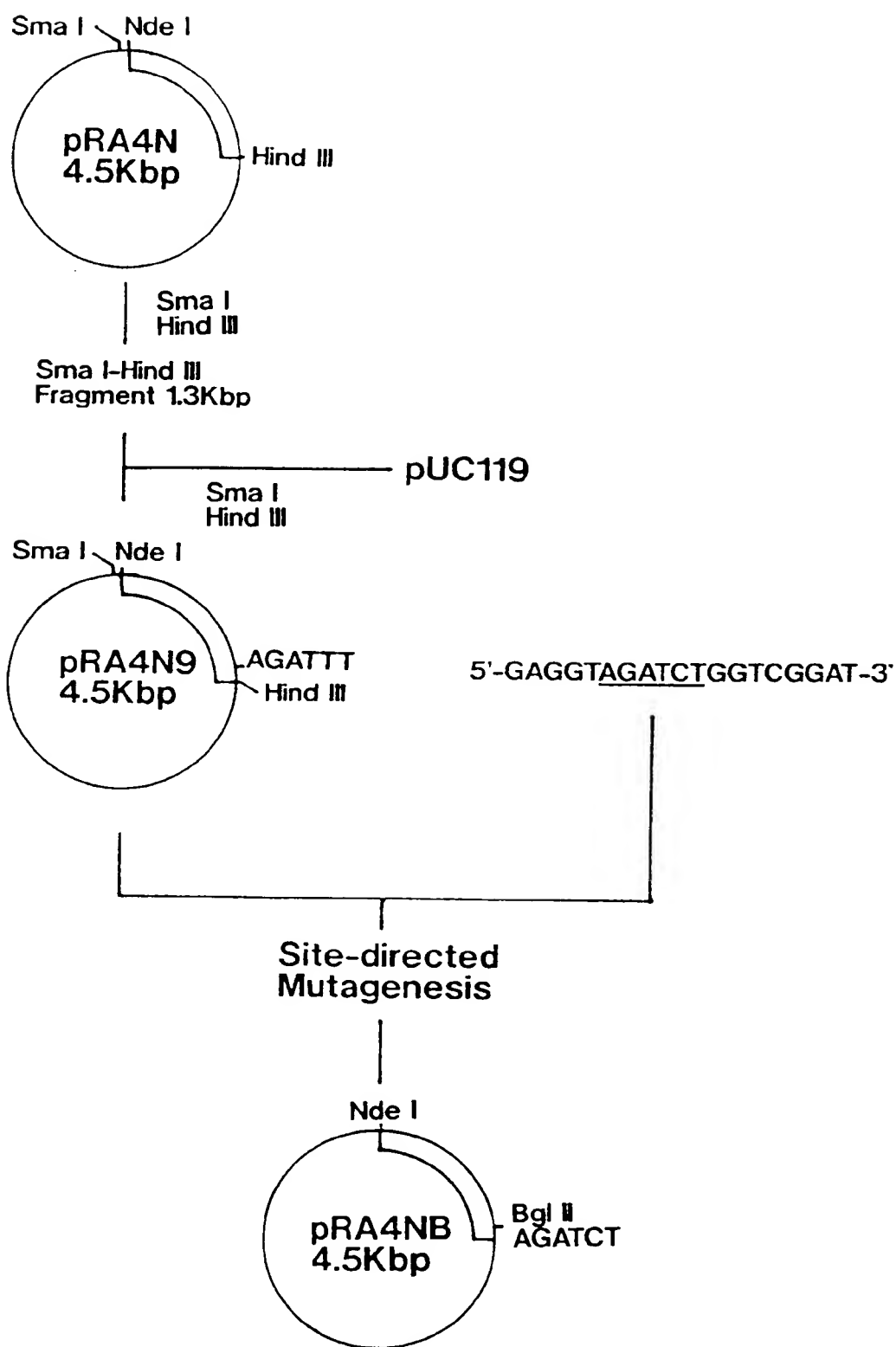
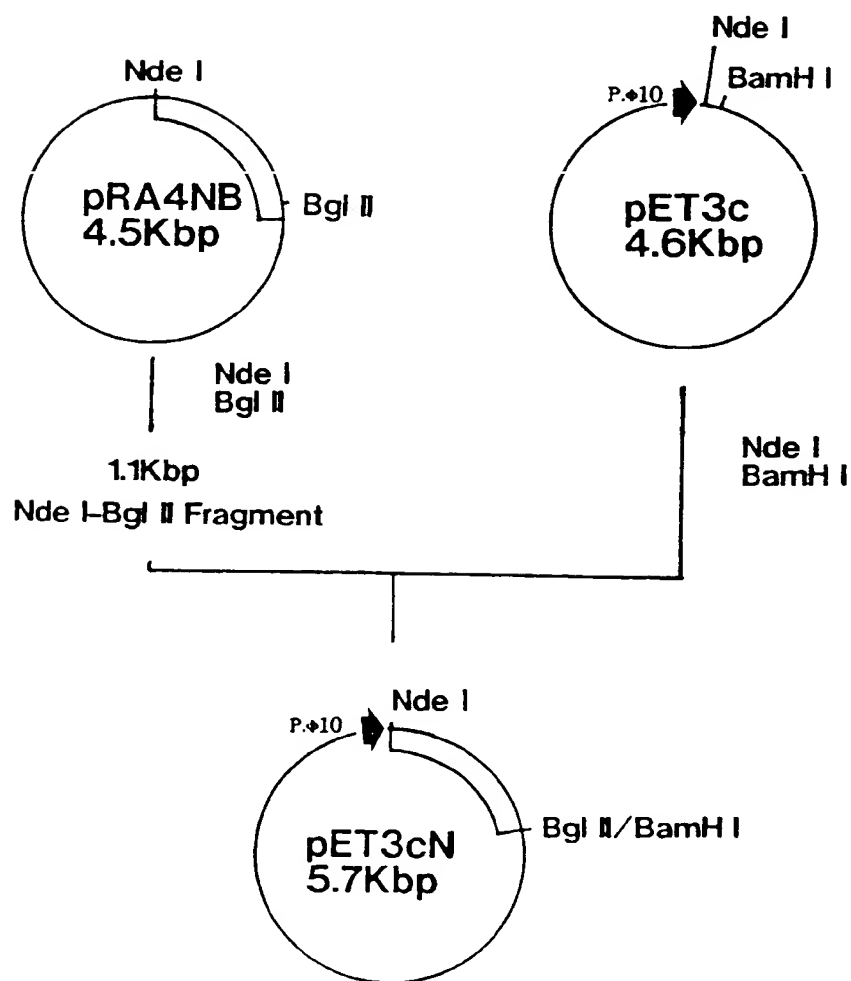


Fig. 9





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(54) **DNA encoding acylamino acid racemase and its use.**

(57) The present invention relates to a DNA fragment containing a gene encoding acylamino acid racemase. The acylamino acid racemase can be produced industrially at high efficiency using the DNA fragment.

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European Patent  
Office

# EUROPEAN SEARCH REPORT

Application Number

EP 91 10 7248

## DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)																								
Y	EP-A-0 304 021 (TAKEDA) 22 February 1989 * claims *	1-3	C12N15/61 C12N1/20																								
D	& US-A-4 981 799 ---																										
Y	CHEMICAL ABSTRACTS, vol. 111, no. 21, 20 November 1989, Columbus, Ohio, US; abstract no. 188773, SODA, KENJI ET AL. 'Plasmid encoding alanine racemase and D-amino acid transaminase.' page 217 ; * abstract * & JP-A-63 304 985 (DAICEL) 13 December 1988 ---	1-3																									
Y	CHEMICAL ABSTRACTS, vol. 107, no. 7, 17 August 1987, Columbus, Ohio, US; abstract no. 53505c, YANAI, AKIRA ET AL. 'Cloning and expression of alpha-amino epsilon-caprolactam racemase gen of Achromobacter obae in Escherichia coli.' page 231 ; * abstract * & JP-A-61 202 693 (TORAY) 8 September 1986 -----	1-3	TECHNICAL FIELDS SEARCHED (Int. Cl.5)  C12N C12P																								
The present search report has been drawn up for all claims																											
Place of search THE HAGUE		Date of completion of the search 23 FEBRUARY 1993	Examiner DELANGHE L.L.M.																								
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